Differential neuroprotective and antiinflammatory effects of estrogen receptor (ER)α and ERβ ligand treatment

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Treatment with either estradiol or an estrogen receptor (ER)α ligand has been shown to be both antiinflammatory and neuroprotective in a variety of neurological disease models, but whether neuroprotective effects could be observed in the absence of an antiinflammatory effect has remained unknown. Here, we have contrasted effects of treatment with an ERα vs. an ERβ ligand in experimental autoimmune encephalomyelitis, the multiple sclerosis model with a known pathogenic role for both inflammation and neurodegeneration. Clinically, ERα ligand treatment abrogated disease at the onset and throughout the disease course. In contrast, ERβ ligand treatment had no effect at disease onset but promoted recovery during the chronic phase of the disease. ERα ligand treatment was antiinflammatory in the systemic immune system, whereas ERβ ligand treatment was not. Also, ERα ligand treatment reduced CNS inflammation, whereas ERβ ligand treatment did not. Interestingly, treatment with either the ERα or the ERβ ligand was neuroprotective, as evidenced by reduced demyelination and preservation of axon numbers in white matter, as well as decreased neuronal abnormalities in gray matter. Thus, by using the ERβ selective ligand, we have dissociated the antiinflammatory effect from the neuroprotective effect of estrogen treatment and have shown that neuroprotective effects of estrogen treatment do not necessarily depend on antiinflammatory properties. Together, these findings suggest that ERβ ligand treatment should be explored as a potential neuroprotective strategy in multiple sclerosis and other neurodegenerative diseases, particularly because estrogen-related toxicities such as breast and uterine cancer are mediated through ERα.

experimental autoimmune encephalomyelitis | neuroprotection | multiple sclerosis selective estrogen receptor modulators

Estrogen treatment has been effective in numerous neurodegenerative disease models, including multiple sclerosis (MS), Parkinson's disease, spinal cord injury, cerebellar ataxia, Down's syndrome, epilepsy, and some models of stroke and Alzheimer's disease (1–4), and translational work using estrogen treatment for human neurodegenerative diseases has begun. In general, there has been somewhat of a disparity in results of estrogen treatment of animal models and results in humans, with excellent results in the former and controversial effects in the latter. In reviewing the possible reasons for the disparity, a "healthy cell bias of estrogen action" has been hypothesized (5). Briefly, efficacy of estrogen treatment appears to depend critically on its administration early, as a preventative therapy, before neurodegeneration has occurred (6). Also, early timing of treatment appears to be important, with respect not only to intervention into the neurodegenerative process but also to the need to avoid a period of hypoestrogenicity. In the Women's Health Initiative study, which showed that estrogen treatment afforded no benefit for stroke prevention, women were postmenopausal for many years before initiating estrogen treatment (7). Recently, it has been shown in an ischemic stroke model that estradiol treatment is effective if administered immediately but not 10 weeks after ovariectomy (8). Based on this knowledge, trials are now being designed that will consider the disease duration and menopausal status of the subjects (9).

Unresolved issues in the strategy to use estrogens as neuroprotective agents include whether neuroprotective effects are secondary to antiinflammatory effects of estrogens, and which estrogen receptor mediates each of these protective properties. Although a variety of antiinflammatory mechanisms of estrogen treatment have been described (10–12), these are not mutually exclusive of more direct neuroprotective mechanisms, because estrogens are lipophilic, readily traversing the blood–brain barrier (13). Further neuroprotective effects of estrogen treatment in neuronal cultures and other *in vitro* systems devoid of an inflammatory confound have been described (14–16). Regarding estrogen receptors, the actions of estrogen are mediated primarily by nuclear estrogen receptor (ER)α and ERβ, although nongenomic membrane effects have been described (17). ERα and ERβ have partially distinct tissue distributions (18), thereby providing for some tissue selectivity using selective estrogen receptor modifiers. The two receptors act synergistically in some tissues, whereas they act antagonistically in others. These tissue-specific differences in biologic outcomes are thought to be due to tissue-specific differences in transcription factors, which become activated on binding of each ER by ligand (19, 20). Despite the fact ERβ has been shown to be expressed widely in the CNS in adult mice (21, 22), in most neurological disease models, the protective effect of estrogen treatment has been shown to be mediated through ERα and has been associated with antiinflammatory effects (8, 21, 23, 24).

Here, we will contrast effects of treatment with an ERα vs. an ERβ ligand in experimental autoimmune encephalomyelitis (EAE), a MS model with a known pathogenic role for both inflammation and neurodegeneration. Results using the ERβ-selective ligand permit one to dissociate the antiinflammatory from the neuroprotective effect of estrogen treatment and demonstrate that neuroprotective effects of estrogen treatment do not necessarily depend on antiinflammatory properties.

Results

Selected Doses of ERα and ERβ Ligands Induced Known Biological Responses on a Positive Control Tissue, the Uterus. Before beginning EAE experiments, we used the uterine response to assess whether a known *in vivo* response would occur during treatment with each

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Abbreviations: EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; DPN, diarylpropionitrile; MBP, myelin basic protein; MS, multiple sclerosis; KO, knockout.

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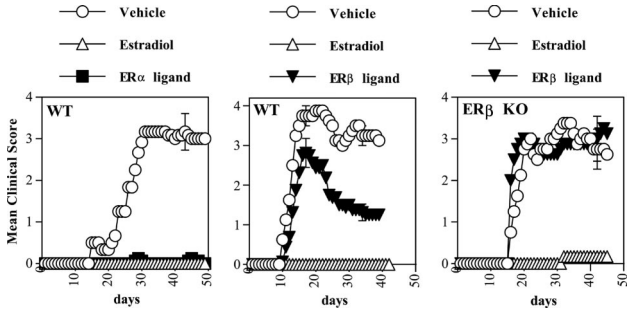

Fig. 1. Treatment with ERα- vs. ERβ-selective ligands has differential effects on chronic EAE. Ovariectomized C57BL/6 female mice were given daily s.c. injections of an ER ligand during active EAE and graded for clinical disease severity using the standard EAE grading scale. (*Left*) Mean clinical scores of ERα ligand-treated mice, as compared with vehicle-treated mice, were significantly reduced throughout disease, $P < 0.0001$. Each treatment group, $n = 4$; data are representative of a total of five repeated experiments. (*Center*) ERβ ligand-treated mice, as compared with vehicle-treated mice, were not significantly different early in disease (up to day 20 after disease induction) but then became significantly improved later during EAE (day 30 after disease induction), $P < 0.001$. Numbers of mice in each group were vehicle, $n = 4$; estradiol, $n = 4$; DPN, $n = 8$. Data are representative of experiments repeated twice. (*Right*) DPN treatment *in vivo* during EAE remains highly selective for ERβ. Clinical scores in ovariectomized ERβ KO C57BL/6 mice with active EAE were no different when comparing DPN-treated with vehicle-treated mice. Each treatment group, $n = 4$, and data are representative of experiments repeated twice. Estradiol-treated mice served as a positive control for a treatment effect in each experiment.

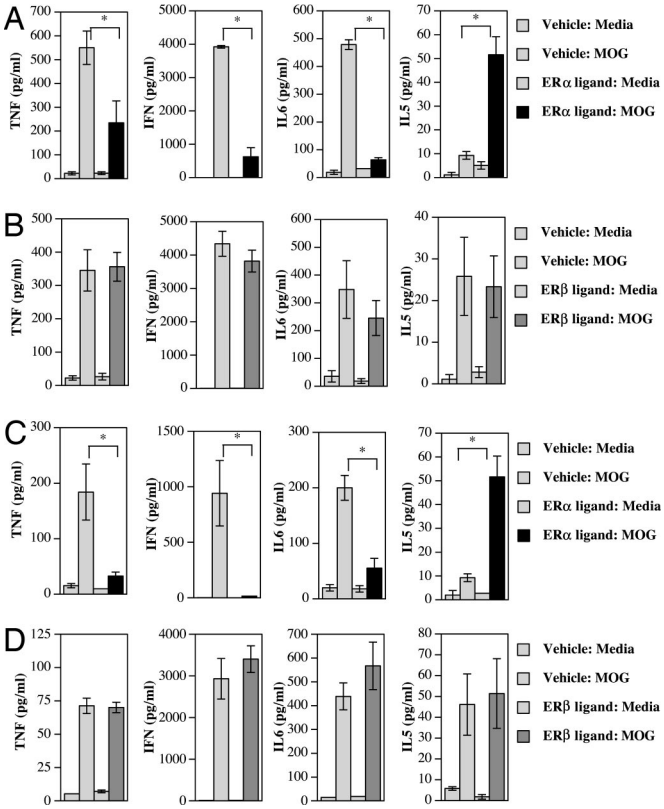

Fig. 2. Treatment with ERα- vs. ERβ-selective ligands has differential effects on the systemic immune response. At day 19 (*A* and *B*) or day 40 (*C* and *D*) after disease induction, mice were killed, and cytokine production by autoantigen-stimulated splenocytes was determined. ERα ligand treatment significantly reduced TNFα, IFN-γ, and IL-6, and increased IL-5, during early EAE (*A*) and late EAE (*C*). In contrast, no significant differences with ERβ ligand treatment were seen in cytokine levels at either the early stage (*B*) or late stage (*D*) of EAE. Vehicle-treated and media-stimulated (first bar) and ER ligand-treated and media-stimulated (third bar) each served as negative controls for stimulations with autoantigen MOG 35–55 peptide (MOG) (second and fourth bars). Error bars indicate variability of cytokine values for individual mice within a given treatment group, with $n = 4$ mice for each treatment group. Data are representative of two to five experiments for each time point. No differences were observed with either ERα or ERβ ligand treatment, as compared with vehicle, for either IL-17 or IL-10, whereas IL-4 and IL-12 levels were too low to detect (not shown).

of our dosing regimens for the ERα and ERβ ligands. It was known that estrogen treatment increased uterine weight primarily through ERα (25), and it had also been shown that treatment with the ERβ ligand diarylpropionitrile (DPN) could antagonize the ERα-mediated increase in uterine weight (26). Thus, we administered the ERα ligand, propyl pyrazole triol, to ovariectomized C57BL/6 females for 10 days at either an optimal (10 mg/kg per day) or suboptimal (3.3 mg/kg per day) dose and observed a significant increase in uterine weight as compared with vehicle treated mice [supporting information (SI) Fig. 8]. When an ERβ ligand dose (8 mg/kg per day) (27) was given in combination with the ERα ligand, the increase in uterine weight mediated by ERα ligand treatment was significantly reduced. These data demonstrated that our method and dose of delivery of the ERα and ERβ ligands induced a known biological response *in vivo* on a positive control tissue, the uterus.

Differential Effects of Treatment with ERα and ERβ Ligands on Clinical EAE. We compared and contrasted effects between ERα and ERβ treatment during EAE. When the ERα ligand was administered 1 week before active EAE induction with myelin oligodendrocyte glycoprotein (MOG) 35–55 peptide in ovariectomized C57BL/6 female mice, clinical disease was completely abrogated ($P < 0.0001$; Fig. 1 *Left*). This was consistent with our previous findings in this EAE model (23), as well as findings in adoptive EAE in SJL mice by others (28). In contrast, ERβ ligand treatment had no significant effect early in disease (up to day 20 after disease induction) but then demonstrated a significant protective effect later in disease (after day 20), $P < 0.001$ (Fig. 1 *Center*).

Our data showing a protective effect using the ERβ ligand DPN in active EAE in C57BL/6 mice were surprising given that another ERβ ligand (WAY-202041) was shown to have no effect in EAE (28), albeit using a different strain of mice that were followed for a shorter time period. Because WAY-202041 was shown to have a 200-fold selectivity for ERβ, whereas DPN has a 70-fold selectivity (29), it was possible that DPN was not sufficiently selective for ERβ *in vivo* in our studies. To assess the *in vivo* selectivity of DPN treatment during EAE, we administered DPN to homozygous ERβ knockout (KO) mice. When DPN was administered to ovariectomized ERβ KO C57BL/6 mice with EAE, the treatment was no longer protective (Fig. 1 *Right*). These data demonstrated the *in vivo* selectivity of DPN for ERβ during EAE at the dose used in our studies.

Together, these results indicated that treatment with an ERα ligand is protective at the acute onset and throughout the course of EAE, whereas treatment with an ERβ ligand is protective during the later phase of the disease, after the acute initial phase.

Differential Effects of Treatment with ERα and ERβ Ligands on Autoantigen-Specific Cytokine Production. Because estrogen treatments in EAE had previously been associated with either a down-regulation of proinflammatory cytokines or an up-regulation of antiinflammatory cytokines (10, 11), we next assessed autoantigen-specific cytokine production by systemic immune cells during ERα vs. ERβ ligand-treated EAE. ERα ligand treatment significantly reduced levels of cytokines (TNFα, IFN-γ, and IL-6) known to be proinflammatory in EAE, whereas it increased the antiinflammatory cytokine IL-5, during both early (Fig. 2*A*) and later (Fig. 2*C*) stages of EAE. In contrast, ERβ ligand treatment was no different

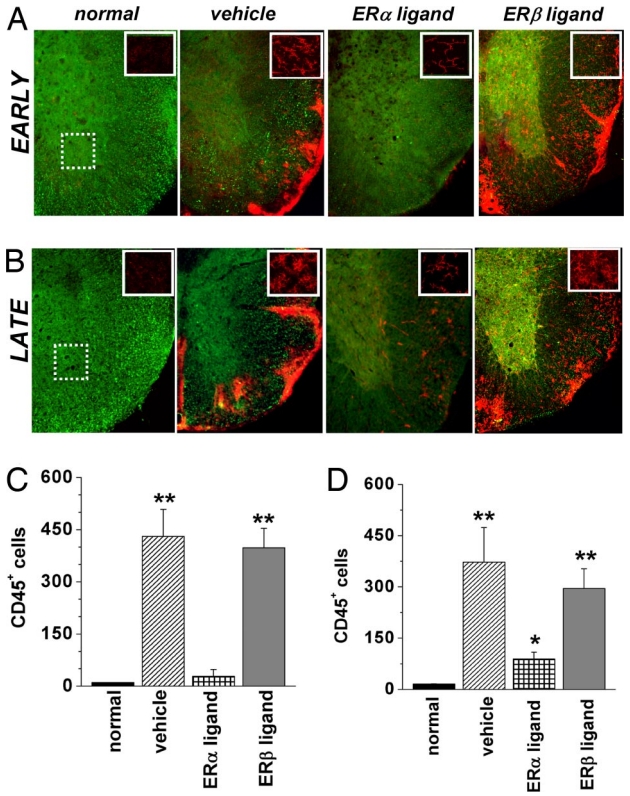

Fig. 3. Treatment with an ERα ligand, not an ERβ ligand, reduced inflammation in spinal cords of mice with EAE. Consecutive thoracic spinal cord sections coimmunostained with NF200 (green) and CD45 (red) at ×10 magnification are shown from partial images (lateral funiculus, a portion of anterior funiculus and gray matter) from normal control, vehicle-treated EAE, ERα ligand-treated EAE, and ERβ ligand-treated EAE mice at day 19 (*A*) and day 40 (*B*) after disease induction. Vehicle-treated EAE cords had large areas of CD45+ cell staining in white matter as compared with the normal control, whereas ERα ligand-treated EAE mice had only occasional CD45 positivity. ERβ ligand-treated EAE mice had CD45+ cell staining, similar to that in vehicle-treated EAE. Consecutive sections from the same mice were also scanned at ×40 magnification (within ventral horn designated by the dotted line square area in normal image) to show the morphology of CD45+ cells in the gray matter. (*C*) Counting CD45+DAPI+ cells in the dorsal funiculi revealed that vehicle- and ERβ ligand-treated EAE mice had a significant increase compared with healthy controls, whereas the ERα ligand-treated groups did not. (*D*) Number of CD45+DAPI+ cells during later EAE was quantified as in *C*. Number of mice, three per treatment group; number of T1–T5 sections per mouse, six; total number of sections per treatment group, 18. Statistically significant compared with normals (*, $P < 0.05$; **, $P < 0.001$), 1 × 4 ANOVAs. Data are representative of experiments repeated in their entirety on another set of EAE mice with each of the treatments.

from vehicle treatment in all measured cytokines (TNFα, IFN-γ, IL-6, and IL-5) at either the early (Fig. 2*B*) or later (Fig. 2*D*) time points. These results indicated that, whereas ERα ligand treatment was antiinflammatory in the systemic immune system, ERβ ligand treatment was not.

Treatment with an ERα Ligand, but Not an ERβ Ligand, Reduces CNS Inflammation. We then addressed whether treatment with ERα vs. ERβ ligands resulted in differences in inflammation within the CNS. At both early (day 19) and later (day 40) stages of EAE, spinal cord sections from mice treated with either vehicle, ERα or ERβ ligand were assessed for inflammation by using anti-CD45 antibody to stain inflammatory cells. ERα ligand-treated EAE compared with vehicle-treated EAE mice had less CD45 staining in white matter. This reduction in CD45 staining was present at both the early (Fig. 3*A*) and later (Fig. 3*B*) timepoints in EAE. In contrast, ERβ ligand-treated EAE mice did not have reduced CD45 staining in white matter, at either time point. Quantification of CD45+ cells revealed that ERα ligand-treated mice at the early stage of EAE had a reduction in inflammation, such that levels were no different as compared with those in normal control mice, whereas CD45+ cell numbers in ERβ ligand-treated EAE mice remained significantly increased and comparable to those in vehicle-treated EAE mice (Fig. 3*C*). At the later time point, quantification detected some inflammation in ERα ligand-treated EAE mice, whereas inflammation in ERβ ligand-treated remained very high and similar to vehicle-treated EAE mice (Fig. 3*D*).

Additionally, CD45 staining of cells in gray matter of vehicle-treated EAE mice was observed at both the early and later time points, with these cells demonstrating a morphology suggestive of activated microglia (Fig. 3 *Insets*). This gray matter inflammation was also decreased with ERα ligand but not ERβ ligand treatment.

H&E staining also revealed that vehicle-treated EAE mice had extensive white matter inflammation at both the early (SI Fig. 9*A*) and later (SI Fig. 9*D*) time points, and that this inflammation was reduced by treatment with the ERα but not the ERβ ligand. Further, when anti-CD3 antibody was used to stain T lymphocytes, and anti-Mac 3 antibody was used to stain cells of the macrophage lineage, the infiltrate was shown to be composed of both T cells and macrophage lineage cells. Treatment with the ERα ligand but not the ERβ ligand reduced this T cell and macrophage lineage cell staining at both the early (SI Fig. 9 *B* and *C*) and later (SI Fig. 9 *E* and *F*) time points.

Together, these data indicated that ERα but not ERβ ligand treatment reduced inflammation in the CNS of mice with EAE.

Treatment with Both ERα and ERβ Ligands Reduces Demyelination in White Matter. The degree of myelin loss was then assessed by myelin basic protein (MBP) immunostaining in the dorsal columns of thoracic cords. Extensive demyelination occurred at the sites of inflammatory cell infiltrates in vehicle-treated EAE mice, whereas less demyelination occurred in ERα and ERβ ligand treated mice (Fig. 4*A* and *B*). Quantification of demyelination by density analysis of MBP immunostained spinal cord sections revealed a 32% ($P < 0.01$) and 34% ($P < 0.005$) decrease in myelin density in vehicle-treated EAE mice, at the early and later time points, respectively, as compared with healthy controls (Fig. 4 *C* and *D*). In contrast, myelin staining was somewhat decreased but relatively preserved in both ERα and ERβ ligand-treated mice with no significant difference as compared with healthy controls. Double immunostaining with antibodies to MBP and to 200-kDa neurofilament (NF200) revealed relatively intact red myelin rings around green axons in the ERα and ERβ ligand-treated EAE mice (SI Fig. 10).

Treatment with Both ERα and ERβ Ligands Reduces Axonal Loss in White Matter. Staining with anti-NF200 antibody revealed axonal loss in white matter of vehicle-treated EAE mice at both early and later time points of disease as compared with healthy controls, whereas both ERα and ERβ ligand-treated EAE mice had less axonal loss (Fig. 5*A* and *B*). Quantification of NF200 staining in the anterior funiculus revealed a 49 ± 12% ($P < 0.01$) and 40 ± 8% ($P < 0.005$) reduction in vehicle-treated EAE, at the early and later time points, respectively, as compared with healthy controls (Fig. 5 *C* and *D*), whereas axon numbers in ERα and ERβ ligand-treated EAE mice were not significantly reduced as compared with those in healthy controls.

Treatment with Both ERα and ERβ Ligands Reduces Neuronal Pathology in Gray Matter. Recently, we demonstrated neuronal abnormalities surprisingly early during EAE (day 15), which were prevented by treatment with either estradiol or ERα ligand (23). Here, we asked whether ERβ ligand treatment might preserve neuronal integrity. We used a combination of Nissl stain histology and NeuN/β3-tubulin immunolabeling to identify and semiquantify neurons in gray matter. A decrease in neuronal staining in gray

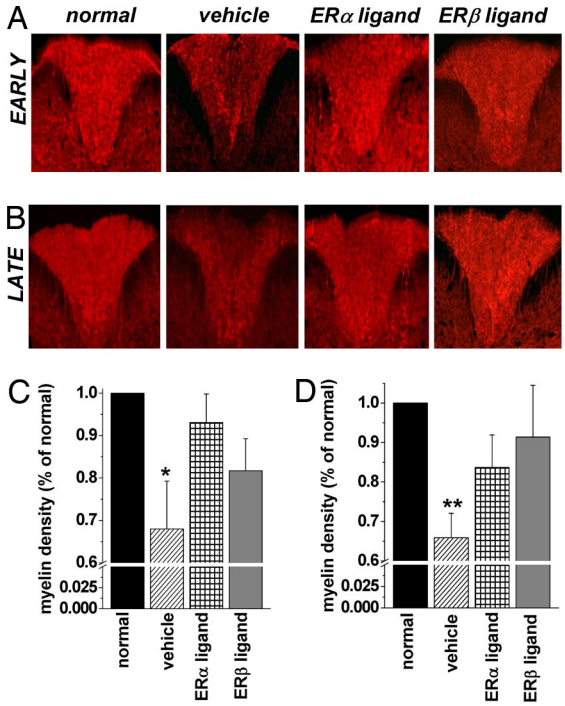

Fig. 4. Treatment with ERα and ERβ ligands, each preserved MBP immunoreactivity in white matter of spinal cords of mice with EAE. At days 19 (*A*) and 40 (*B*) after disease induction, vehicle-treated EAE mice had reduced MBP immunoreactivity as compared with normal controls in dorsal columns of thoracic spinal cord sections imaged at ×10 magnification. In contrast, ERα and ERβ ligand-treated EAE mice showed relatively preserved MBP staining. Upon quantification (*C* and *D*), MBP immunoreactivity in the dorsal column was significantly lower in vehicle-treated EAE mice as compared with normal mice, whereas ERα and ERβ ligand-treated EAE mice each demonstrated no significant decreases. Myelin density is presented as percent of normal. Number of mice, three per treatment group; number of T1–T5 sections per mouse, six; total number of sections per treatment group, 18. Statistically significant compared with normal (*, $P < 0.01$; **, $P < 0.005$); 1 × 4 ANOVAs.

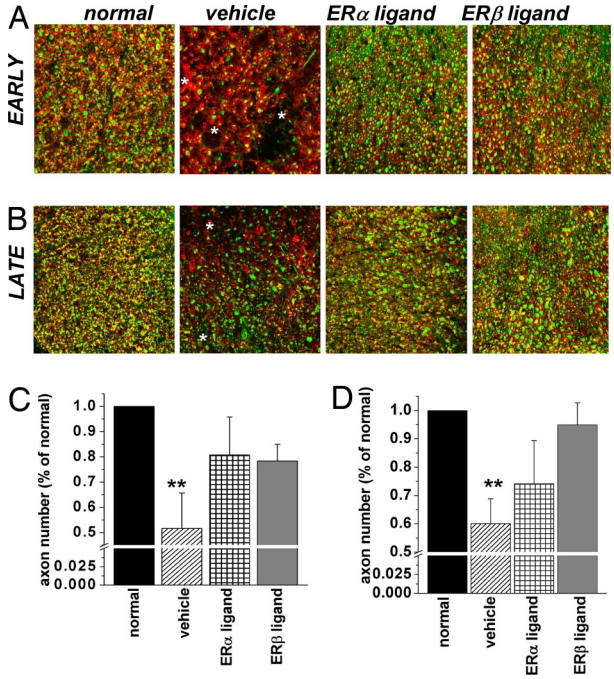

Fig. 5. Treatment with ERα and ERβ ligands each preserved axonal densities in white matter of spinal cords of mice with EAE. Part of the anterior funiculus of thoracic spinal cord sections was imaged at ×40 after coimmunostaining with anti-NF200 (green) and anti-MBP (red). Distinct green axonal centers surrounded by red myelin sheaths can be seen in normal controls, and ERα and ERβ ligand-treated EAE mice at 19 days (*A*) and 40 days (*B*) after disease induction. In contrast, vehicle-treated EAE mice showed reduced axonal numbers and myelin, with focal demyelination (white asterisks). Upon quantification (*C* and *D*), neurofilament-stained axon numbers in white matter were significantly lower in vehicle-treated EAE mice as compared with normal mice, whereas ERα and ERβ ligand-treated EAE mice demonstrated no significant reduction in axon numbers as compared with normal controls. Axon number is presented as percent of normal. Number of mice, three per treatment group; number of T1–T5 sections per mouse, six; total number of sections per treatment group, 18. Statistically significant compared with normal (*, $P < 0.01$; **, $P < 0.005$); 1 × 4 ANOVAs.

matter occurred at both time points in vehicle-treated EAE mice as compared with healthy controls, whereas neuronal staining in gray matter was preserved in EAE mice treated with either the ERα or the ERβ ligand at the early and the later time points (Fig. 6 *A* and *B*). Quantification of NeuN+ cells in gray matter demonstrated a 41 ± 13% ($P < 0.05$) and 31 ± 8% ($P < 0.05$) reduction at the early and later time points, respectively, in vehicle-treated EAE mice as compared with normal controls, whereas ERα and ERβ ligand-treated mice had NeuN+ cell numbers that were fewer but not significantly different from those in healthy controls (Fig. 6 *C* and *D*).

Protection from Neuropathology Is Mediated by ERβ. To confirm whether the effect of DPN treatment *in vivo* on CNS neuropathology was indeed mediated through ERβ, we next assessed neuropathology in DPN-treated EAE mice deficient in ERβ. At day 38 after disease induction, inflammation, demyelination, and reductions in axon numbers were present in white matter, whereas neuronal staining was decreased in gray matter of vehicle-treated EAE mice (SI Fig. 11). In contrast to the neuroprotection observed during DPN treatment of WT mice (Figs. 4–6), DPN treatment of ERβ KO mice failed to prevent this white and gray matter pathology (SI Fig. 11). These data demonstrated that neuroprotective effects mediated by DPN treatment *in vivo* during EAE are mediated through ERβ.

Treatment with an ERβ Ligand Induces Recovery of Motor Performance. Because treatment with an ERβ ligand was found to be neuroprotective in EAE, we then assessed the clinical significance of this neuroprotective effect using an outcome frequently used in spinal cord injury, rotarod performance. Vehicle-treated EAE mice demonstrated an abrupt and consistent decrease in the number of seconds they were able to remain on the rotarod, beginning at day 12 after disease induction, and this disability remained throughout the observation period. ERβ ligand-treated mice also had an abrupt decrease in the number of seconds they could remain on the rotarod. However, later, at days 30–40, they had significant recovery (Fig. 7 *Left*). These data demonstrated that ERβ ligand treatment induced functional recovery in motor performance at later time points during EAE. Finally, the improvement in rotarod performance with DPN treatment was no longer observed in the ERβ KO (Fig. 7 *Right*), demonstrating that the DPN-induced recovery in motor performance later in disease was indeed mediated through ERβ.

Discussion

Previously, it had been shown that treatment with either estradiol or an ERα ligand was antiinflammatory and neuroprotective in EAE, stroke, and other disease models (8, 21, 23, 24). Whether neuroprotective effects could be observed in the absence of an antiinflammatory effect remained unknown, with a recent study suggesting that an antiinflammatory effect was necessary to observe neuroprotection in stroke (8). In this study, we have contrasted effects of treatment with ERα vs. ERβ ligands in EAE, the MS model with a known pathogenic role for both inflammation and

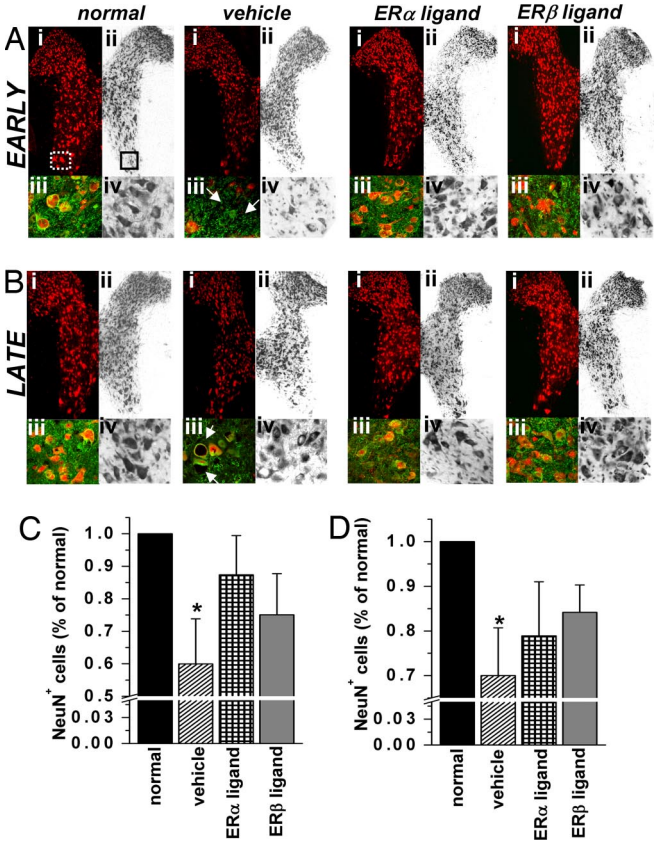

Fig. 6. Treatment with ERα and ERβ ligands each preserved neuronal staining in gray matter of spinal cords of mice with EAE. Split images of thoracic spinal cord sections stained with NeuN (red, *i*) and Nissl (*ii*) at ×4 magnification, derived from healthy controls, vehicle-treated EAE, ERα ligand-treated EAE, and ERβ ligand-treated EAE mice, killed at either day 19 (early; *A*) or day 40 (late; *B*) after disease induction are shown. *iii* is a merged confocal scan at ×40 of NeuN+ (red) and β3-tubulin+ (green) colabeled neurons from an area represented by the dotted white square area in *i*. *iv* is a ×40 magnification of Nissl-stained area in solid black square in *ii*. A decrease in NeuN+ immunostaining and Nissl staining was observed in the dorsal horn, intermediate zone, and ventral horn of vehicle-treated EAE mice as compared with normal control. White arrows in *iii* denote loss of NeuN+ staining. In contrast, EAE mice treated with either ERα or ERβ ligand had preserved NeuN and Nissl staining. Upon quantification of neurons in the entire delineated gray matter of T1–T5 sections, NeuN+ immunolabeled neurons were significantly decreased by nearly 41% in vehicle-treated EAE mice at day 19 (*C*) and nearly 31% at day 40 (*D*) as compared with normal controls, whereas ERα and ERβ ligand-treated EAE mice were not statistically different from normal controls. Number of mice, three per treatment group; number of T1–T5 sections per mouse, six; total number of sections per treatment group, 18. Statistically significant compared with normals (*, $P < 0.05$), 1 × 4 ANOVAs. Data are representative of experiments repeated in their entirety on another set of EAE mice with each of the treatments.

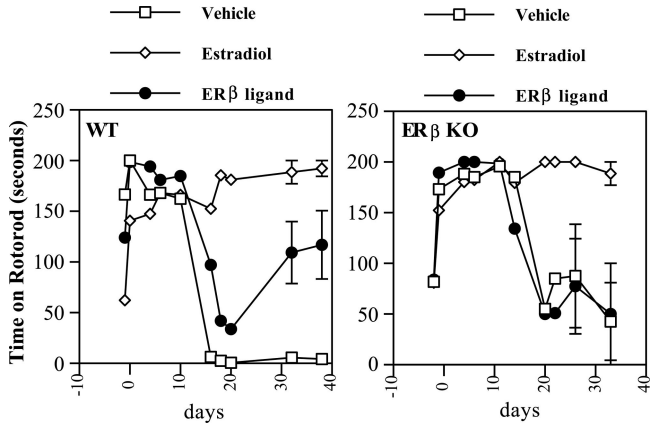

Fig. 7. Treatment with an ERβ ligand results in recovery of motor function late during EAE. (*Left*) Ovariectomized C57BL/6 female mice with EAE were treated with ERβ ligand and assessed for motor performance on a rotarod apparatus. Although mean time on rotarod decreased abruptly at day 12 after disease induction in both the vehicle- and ERβ ligand-treated EAE mice, after day 30, the ERβ ligand-treated group demonstrated significant recovery of motor function, whereas the vehicle-treated did not improve. *, $P < 0.01$ and **, $P < 0.005$, ANOVA. Estradiol treatment served as a positive control for a treatment effect. Number of mice in each treatment group: vehicle, $n = 4$; DPN, $n = 8$; estradiol, $n = 4$. Data are representative of experiments repeated twice. (*Right*) In contrast to the improvement observed with ERβ ligand treatment of WT mice, no improvement was observed at the later phase of disease in ERβ ligand-treated ERβ KO mice. Again, vehicle served as a negative control, and estradiol served as a positive control, for a treatment effect. Number of mice in each treatment group: vehicle, $n = 4$; DPN, $n = 4$; estradiol, $n = 4$.

neurodegeneration. We found that treatment with the ERβ ligand was neuroprotective, with no evidence of an antiinflammatory effect, when assessing both systemic immune responses and CNS inflammation. Thus, by using the ERβ-selective ligand, we have dissociated the antiinflammatory effect from the neuroprotective effect of estrogen treatment and have shown that neuroprotective effects of estrogen treatment do not necessarily depend on antiinflammatory properties. What remains unknown is whether the neuroprotective effect of ERβ ligand treatment is mediated through binding to ERβ receptors on neurons, oligodendrocytes, or astrocytes, because all of these CNS cell types have been shown to express ERβ *in vivo* (30).

It was interesting to contrast the effects of ERα vs. ERβ ligand treatment. Treatment with the ERα ligand, which was antiinflammatory, resulted in complete abrogation of the onset of EAE and thereafter. In contrast, treatment with the ERβ ligand, which was not antiinflammatory, resulted in no significant effect at the onset of disease but induced clinical protection later in disease. On rotarod testing, motor performance was severely impaired initially but then underwent recovery in the ERβ ligand-treated mice. Finally, ERβ ligand-treated mice had extensive inflammation yet demonstrated significant preservation of axons and neurons throughout the disease course. Together, these data suggested that early treatment targeted toward preserving axonal and neuronal integrity changed the ultimate result of a given inflammatory attack, from permanent disability in untreated, to temporary disability in treated.

To our knowledge, the only previously described neuroprotective agents for EAE, which did not decrease CNS inflammation, were blockers of glutamate receptors (31, 32). These treatments resulted in a modest reduction in neurologic impairment, and the effect was lost after cessation of treatment (32, 33). Glutamate blockers are currently used in amyotrophic lateral sclerosis and Alzheimer's disease with modest success. In MS, brain atrophy on MRI has been detected at the early stages of disease (34), thus a neuroprotective agent would need to be started relatively early, generally at ages 20–40 years, and continued for decades. Because glutamate is needed for normal neuronal plasticity and memory (35), treatment of relatively young individuals with glutamate blockers for decades may be associated with significant toxicity (36). Hence, the identification of an alternative neuroprotective agent represents an important advance in preclinical drug development in MS and other chronic neurodegenerative diseases.

One must consider the risk/benefit ratio of any estrogen treatment when considering its use in neurodegenerative diseases. The goal is to optimize efficacy and minimize toxicity. Hence, determining which estrogen receptor mediates the neuroprotective effect of estrogen treatment is of central importance. Our data demonstrating that treatment with an ERβ ligand is neuroprotective are of clinical relevance, because breast and uterine endometrial cancer

are both mediated through ERα, not ERβ. For neurodegenerative diseases with only a minimum inflammatory component, treatment with an ERβ ligand may suffice. For diseases such as MS with a significant inflammatory component, a standard antiinflammatory treatment could be used in combination with ERβ ligand treatment. In each of these scenarios, the neuroprotective properties of estrogen treatment could be maintained while avoiding the increased risk of cancer in the breast and uterus.

Materials and Methods

Animals. Female WT C57BL/6 mice and ERβ homozygous KO mice on the C57BL/6 background, age 8 weeks, were obtained from Taconic Farms (Germantown, NY). Animals were maintained in accordance with guidelines set by the National Institutes of Health and as mandated by the University of California Los Angeles Office for the Protection of Research Subjects and the Chancellor's Animal Research Committee.

Reagents. Propyl pyrazole triol and diarylpropionitrile (DPN), ERα and ERβ agonists, respectively, were purchased from Tocris Bioscience (Ellisville, MO). Estradiol was purchased from Sigma–Aldrich (St. Louis, MO). Miglyol 812 N liquid oil was obtained from Sasol North America (Houston, TX). MOG peptide, amino acids 35–55, was synthesized to >98% purity by Mimotopes (Clayton, Victoria, Australia).

Hormone Manipulations and EAE Induction. Ovariectomized mice were treated with daily s.c. injections of estradiol at 0.04 mg/kg per day (37), DPN at 8 mg/kg per day (27), propyl pyrazole triol at 10 mg/kg per day (25), or vehicle beginning 7 days before EAE induction and throughout the entire disease duration. Active EAE was induced by immunizing with 300 μg of MOG peptide, amino acids 35–55, and 500 μg of *Mycobacterium tuberculosis* in complete Freund's adjuvant as described (10), and mice were monitored daily for clinical signs as described in *SI Text*. Some mice were followed clinically for up to 50 days after disease induction, whereas others were killed earlier for mechanistic studies at day 19 after disease induction, corresponding to days 4–6 after the onset of clinical signs in the vehicle-treated group. Uterine weights to assess the biological response to dosing were as described in *SI Text*.

Rotarod Testing. Motor behavior was tested up to two times per week for each mouse using a rotarod as described in *SI Text*.

Immune Responses. Splenocytes were stimulated with autoantigen at 25 μg/ml, supernatants were collected after 48 and 72 h, and levels of TNFα, IFN-γ, IL-6, and IL-5 were determined by cytometric bead array (BD Biosciences, San Diego, CA), as described (10), and IL-17 was measured by ELISA (R&D Systems, Minneapolis, MN)

Histologic Preparation and Immunohistochemistry. Perfusion and spinal cord collections were carried out as described in *SI Text*. Serial sections were stained with H&E or Nissl.

Consecutive sections were also examined by immunohistochemistry (23) by using primary antibodies: anti-β3 tubulin and anti-neurofilament-NF200, anti-neuronal specific nuclear protein (NeuN), anti-CD45, and anti-MBP [Chemicon (Temecula, CA) and Sigma], as described in *SI Text*.

Microscopy and Quantification. Sections from spinal cord levels T1–T5 were examined, six from each mouse, with $n = 3$ mice per treatment group, for a total of 18 sections per treatment group. Images were captured under microscope (×4, ×10, or ×40) by using the DP70 Image software and a DP70 camera (both from Olympus, Melville, NY). All images were converted to grayscale and then analyzed by density measurement with ImageJ, ver. 1.29 (http://rsb.info.nih.gov/ij). Increase in total number of infiltrating cells was measured by density measurements of DAPI+ nuclei in the whole white matter. Neuronal cells were quantified by counting the NeuN+/β3-tubulin+/DAPI+ cells per mm^2 in the whole gray matter. Laser-scanning confocal microscopic scans were performed on MBP+/NF200+ and CD45+/NF200+ immunostained spinal cord sections, each as described in *SI Text*.

Statistical Analysis. EAE clinical disease severity was compared between treatment groups by using the Friedman test; histopathological changes were assessed by using 1 × 4 ANOVAs; uterine weights, proliferative responses, and cytokine levels were compared between treatment groups using Student's t test, and time on rotorod was compared between treatment groups by using ANOVA.

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